Re-evaluation of the Current NMI01 STR Sizing System of *Cannabis* DNA

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Introduction

The NMI01 STR region of *Cannabis sativa* DNA is currently used for source attribution of seized *Cannabis* by law enforcement.[1] However, the current system does contain some limitations, mainly the lack of a currently used allelic ladder in conjunction with the GeneScan 500 internal size standard. As part of an extended study on the stability of plant DNA on evidence collection cards, FTA cards with seized *Cannabis* from 2009 and 2012 and fresh samples were amplified using CS1F and CS1R primers. Overall, the alleles 2, 16.1, 22.1, 23, 26, 28, and 30.1 were chosen to include in the proposed custom NMI01 allelic ladder in this study. The current NMI01 bin set is calibrated to the GeneScan 500 ROX size standard, which contains a possible sizing gap due to its temperature dependent 250 bp peak.[2] Overall, the GeneScan 500 size standard had more precise fragment sizing than the GeneFlo 625 in the inter-run analysis and similar sizing precision in the intra-run analysis. Despite the fact that most of the alleles were called above 90% accuracy with the GeneScan 500 standard, the allele 28 was only accurately called 77.38% of the time. Additionally, the sizing program Open Source Independent Review and Integration System (OSIRIS) was modified for the NMI01 bin sets as it contains its own custom-sizing algorithm.

Objectives

I. Analyze NMI01 STR region of *Cannabis* treated FTA cards from 2009 and 2012 and fresh samples to obtain the STR profiles
II. Select possible alleles for allelic ladder based on amplification efficiency and peak balance and generate custom allelic ladder
III. Analyze the intra-run and inter-run sizing precision of the NMI01 STR system using both GeneScan 500 and GeneFlo 625 size standards
IV. Re-calibrate the NMI01 sizing system to OSIRIS software

Materials and Methods

FTA cards treated with *Cannabis* from DEA drug seizures in 2009 and 2012 as well as cards treated with seized *Cannabis* from the UNH Police were chosen for DNA analysis. A 3mm hole punch was taken out of each FTA card before undergoing a washing procedure using TE[-1] buffer and FTA Purification Reagent. The resulting FTA cards were amplified using the Terra PCR Direct Genotyping Kit as well as CS1F and CS1R primers.[3] The alleles 2, 16.1, 22.1, 23, 26, 28, and 30.1 were chosen to include in the proposed custom NMI01 allelic ladder in this study. Samples from the previous amplifications with theses alleles were chosen for the ladder based on amplification efficiency and peak balance. The resulting amplicons were combined in solution following the necessary dilutions to generate a 100 uL stock ladder. The custom allelic ladder was analyzed by both inter- and intra-run fragment analysis in triplicate on an ABI 3130xl Genetic Analyzer. Following this, the sizing software OSIRIS was modified for the NMI01 system and samples were analzyed to simulate real-life case-work.

Results

I. Analyze NMI01 STR Profiles

A.

Sample ('12)	Allele	Size (bp)	Height (RFU)
Box 21 #22	16.1	212.94	3350
	22	248.33	2074
Box 21 #25	16.1	212.74	5138
	22	248.15	2790
Box 17 #083	22.1	248.52	3555
	23	253.99	4074
Box 17 #084	11.2	183.83	7446
	26	271.82	2541
Box 20 #088	23	253.69	2544
	26	271.72	2470
Box 18 #086	16.1	212.75	1456
	26	271.66	975
Box 16 #080	16.1	212.72	2124
	25.5	271.41	1374

B.

Sample ('09)	Allele	Size (bp)	Height (RFU)
RW09	1.5	127.42	496
	17.1	218.56	580
	30	296.09	451
CPMS09	16	211.69	71
CPLS09	30.1	296.59	8193

C.

Sample (Fresh)	Allele	Size (bp)	Height (RFU)
4AA	26	272.23	6215
4A	16.1	212.99	7575
	28	283.52	5099
SD	2	127.79	8084
	22.1	248.63	2415
6F	25.5	271.21	881
	27.4	282.19	1028

Table 1. Profiles from Cannabis treated FTA cards from '12 (A), '09 (B), and fresh samples (C) utilizing the GeneScan 500 for fragment sizing. Highlighted samples indicate those utilized to develop the custom NMI01 allelic ladder.
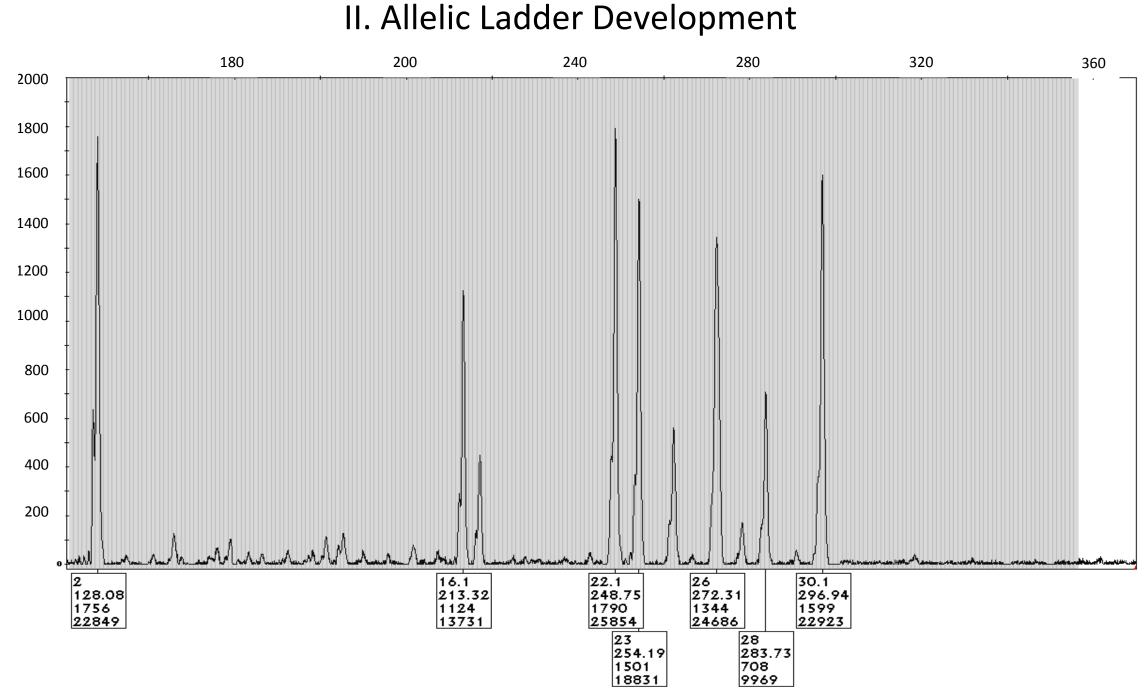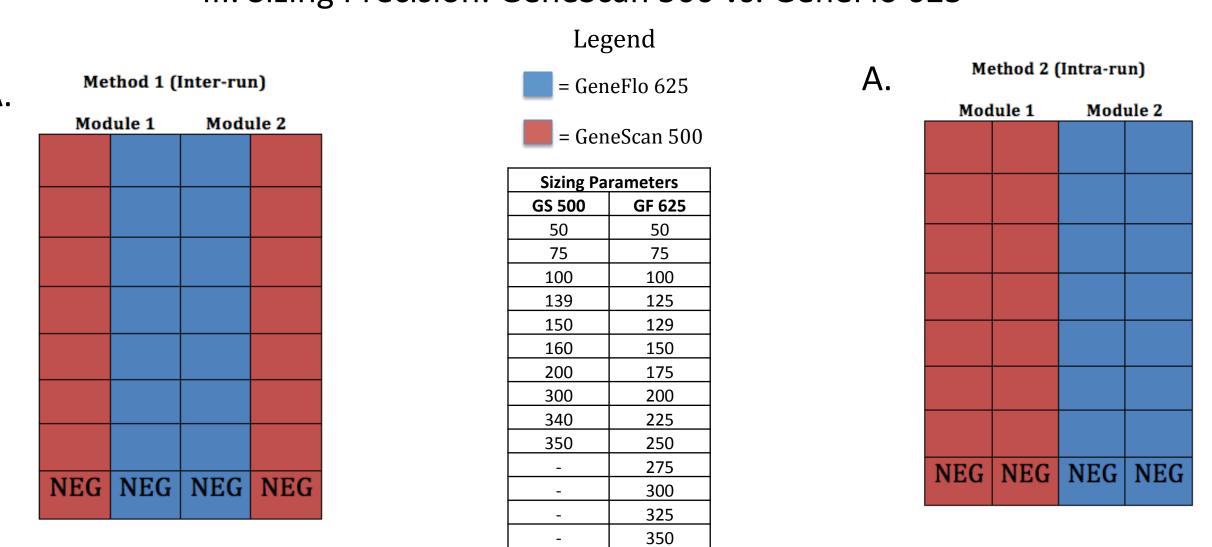
Results

II. Allelic Ladder Development

Figure 1. Electropherogram of custom NMI01 allelic ladder

III. Sizing Precision: GeneScan 500 vs. GeneFlo 625

Sizing Parameters	
GS 500	GF 625
50	50
75	75
100	100
139	125
150	129
160	150
200	175
300	200
340	225
350	250
-	275
-	300
-	325
-	350

B.

GS 500	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6	Allele 7
Average Size (bp)	128.10	213.29	248.70	254.17	272.28	283.70	296.91
SD	0.0949	0.1217	0.1437	0.1521	0.1890	0.1855	0.2283
SE	0.0146	0.0188	0.0222	0.0235	0.0292	0.0286	0.0352

Data Points (GS 500): 42

GF 625	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6	Allele 7
Average Size (bp)	129.75	213.87	250.98	256.68	275.26	286.27	299.02
SD	0.0690	0.1061	0.2792	0.2901	0.3368	0.2746	0.2801
SE	0.0106	0.0164	0.0431	0.0448	0.0520	0.0424	0.0432

Data Points (GF 625): 42

Table 2. Diagram depicting sample placement for inter-run experiment (A). Data outlining the average fragment size, SD, and SE for each allele in the allelic ladder (B).

B.

GS 500	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6	Allele 7
Average Size (bp)	127.98	213.22	248.65	254.10	272.24	283.64	296.88
SD	0.0855	0.1303	0.1667	0.1638	0.1914	0.1947	0.2274
SE	0.0132	0.0201	0.0257	0.0253	0.0295	0.0301	0.0351

Data Points (GS 500): 41

GF 625	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6	Allele 7
Average Size (bp)	129.59	213.63	250.50	256.19	274.71	285.81	298.56
SD	0.0443	0.0595	0.1031	0.1090	0.1533	0.0999	0.0999
SE	0.0069	0.0093	0.0161	0.0170	0.0239	0.0156	0.0156

Data Points (GF 625): 42

Table 3. Diagram depicting sample placement for intra-run experiment (A). Data outlining the average fragment size, SD, and SE for each allele in the allelic ladder (B).
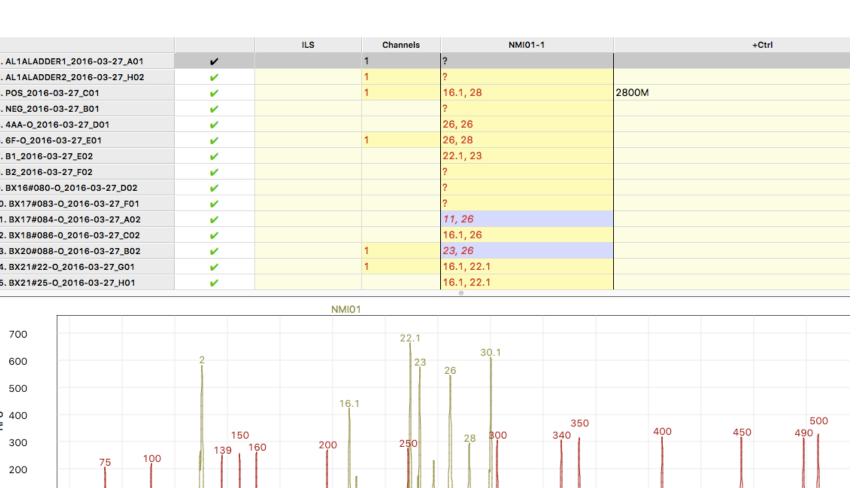
IV. OSIRIS Sizing Software

Figure 2. Case-work module ran with NMI01 allelic ladder and analyzed via OSIRIS software.

Sample	Box 21 #22	Box 21 #25		6F	4AA	Box 17 #084	Box 20 #088
Data Points	11	12	12	12	12	12	12
Allele(s)	22.1	16.1	22.1	16.1	26	28	26	11.1	26	23	26
Average Size	212.97	248.99	212.96	248.97	272.36	283.97	271.96	182.57	271.92	254.03	271.88
SD	0.0548	0.0935	0.0835	0.1288	0.0791	0.0612	0.0494	0.0565	0.0724	0.0791	0.0849
SE	0.0165	0.0282	0.0241	0.0372	0.0228	0.0177	0.0143	0.0163	0.0209	0.0228	0.0245

Table 4. Sizing precision for samples analyzed with OSIRIS using the custom allelic ladder.

Sample	Box 21 #22	Box 21 #25		6F	4AA	Box 17 #084	Box 20 #088
Data Points	12	12	12	12	12	12	12
Allele(s)	22.1	16	22.1	16	26	27.5	26	11.2	26	23	26
Average Size	212.71	248.18	212.61	248.06	271.96	283.01	271.65	183.52	271.59	253.59	271.52
SD	0.0762	0.0652	0.0427	0.0567	0.0766	0.0850	0.0748	0.1085	0.0761	0.0792	0.0679
SE	0.0220	0.0188	0.0123	0.0164	0.0221	0.0245	0.0216	0.0313	0.0220	0.0229	0.0196

Table 5. Sizing precision for samples analyzed with GeneMapper.

Sizing Software	Correct Allele Calls	Allele Calls	Allele Call Accuracy (%)	P-value
OSIRIS	128	130	98.5	P<0.001
GeneMapper	122	132	92.3	

Table 6. Allele call accuracy for samples was statistically significant when comparing results between OSIRIS and GeneMapper.

Discussion

Cannabis treated FTA cards from both 2009 and 2012 all gave NMI01 profiles following extraction, amplification, separation and detection. Even the sample SD, which was a dry plant sample from 2005, was able to obtain a NMI01 profile following FTA card treatment. These results support the concept of using FTA cards as a long-term storage device of *Cannabis* DNA in forensic casework.

Overall, the GeneScan 500 size standard had more precise fragment sizing than the GeneFlo 625 in the inter-run analysis and similar sizing precision in the intra-run analysis. These results agree with past research that has shown the number of fragments in an internal size standard is not a significant factor in sizing precision.[4] Of particular interest, the GeneScan 500 size standard had consistent standard deviation in fragment sizing between the inter-run and intra-run samples. Despite the high precision (+/- 0.25bp), the GeneScan 500 standard displayed an average sizing quality of 0.8714 across the inter- and intra-run analysis versus the GeneFlo 625 standard sizing quality of 0.9937. As alleles ranging from 14 to 30.4 fall within the 200-300 bp region, an allelic ladder is needed to ensure accurate sizing in the current system.

The OSIRIS software successfully analyzed an entire module simulating real-life casework with allelic ladder samples, a positive control, a negative control and suspected *Cannabis* samples. Of particular interest, the samples Box 21 #22 and Box 21 #25, which were obtained from the Mountain and Valley Marijuana Investigation Team (MAVMIT) in an effort to link common origin, gave identical NMI01 profiles (16.1, 22.1) indicating a common origin. Further, when analyzing *Cannabis* samples, the correct allele call was obtained 98.5% of the time with OSIRIS using the custom allelic ladder, while the current system under GeneMapper had 92.4 % accuracy. The OSIRIS software only failed to call the 11.1 allele in sample Box 17 #084 accurately most likely due to the fact that this allele is not present in the custom allelic ladder. Overall, the open source and relatively mobile nature of OSIRIS will potentially further enhance the law enforcement efforts in tracking illegal *Cannabis* activity.

Conclusions

Due to the effectiveness of STR analysis, there has been a large push to identify additional STR regions in *Cannabis'* DNA in an effort to improve identification accuracy as well as overcome the difficulties present in analyzing *Cannabis* plants generated through clonal propagation.[5] Overall, the GeneScan 500 size standard appears to size amplicons within the accepted sizing precision, but modifying the system for the automated sizing software OSIRIS along with the use of a further comprehensive allelic ladder may help the current system approach 100% accuracy in correct allele assignment.

References

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Acknowledgements